An exploratory study to determine whether infliximab modifies levels of rheumatoid factor and antibodies to cyclic citrullinated peptides in rheumatoid arthritis patients

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Abstract

Objective

The aim of this study was to investigate the relationship between serum infliximab (IFX) levels and changes of RF and ACPA levels in patients with rheumatoid arthritis (RA).

Methods

Enzyme-linked immunosorbent assays (ELISA) [Promonitor® IFX R1 (version 2) (Progenika Biopharma, Spain)] were used to measure drug levels and antidrug-antibodies (ADAb) in IFX RA-treated patients (n=19). Disease activity was assessed using DAS28. IgM rheumatoid factor (RF) and IgM, IgA and IgG anti-cyclic citrullinated peptide (ACPA) were determined through ELISA.

Results

A significant decrease in RF (p=0.01), ACPA IgG (p=0.007), IgM (p=0.01) and IgA (p=0.03) was observed in patients presenting adequate levels of serum IFX. No significant changes to RF or ACPA were observed in patients with undetectable IFX.

Conclusion

Data from this study support the hypothesis that the anti-TNF antagonist IFX downregulates autoantibody levels in RA patients when IFX levels are detectable. Larger-scale studies need to be performed to establish RF and ACPA presence as therapeutic response predictive factors.

Key words

enzyme-linked immunosorbent assay (ELISA), anti-TNF, infliximab, anti-drug antibodies (ADAb), rheumatoid arthritis

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Introduction

Tumour necrosis factor antagonists (anti-TNF) have become one of the most widely used biological therapies for patients with rheumatoid arthritis (RA) (1). Despite impressive overall clinical impact, more than one quarter of patients still fail to achieve satisfactory response rates to these biological agents (2). As these drugs are costly and have side effects, a need for anti-TNF response predictive factors would be welcome. Different study groups have already shown that the lack of response to anti-TNF in some non-responder patients is associated with the development of anti-drug antibodies (ADAb), which have been correlated with lower drug levels in blood (3-7). Rheumatoid factor (RF) and antibodies to citrullinated peptides (ACPA) have also been investigated as serological parameters potentially indicative of drug response in patients treated with anti-TNF, although a clear link is yet to be established (8-11). Remarkably, none of these studies looked at drug levels, the presence of anti-drug antibodies (ADAb) and their relationship with clinical response while considering RA-related autoantibody concentrations. This is the context for our hypothesis: that detectable levels of infliximab modify RF and ACPA concentrations. The aim of this pilot study was to investigate the relationship between serum infliximab (IFX) levels and changes of RF and ACPA levels in patients with RA.

Patients and methods
Patient selection

Nineteen patients fulfilling the American College of Rheumatology (ACR) classification criteria for RA (12) treated with the anti-TNF drug infliximab (IFX, Remicade®, Centocor Ortho Biotech Inc., Malvern, PA, EE.UU) as a first line of biological therapy with standard doses (3.5 mg/kg at week 0, 4, 6, and every 8 weeks) were enrolled in this retrospective study.

All patients were taking IFX as their first biological therapy and underwent infusion every 8 weeks at our day unit with serum samples collected immediately beforehand. In addition, sequential serum samples were taken, frozen at -70ºC and stored in our serum bank. The study used baseline and post-IFX treatment samples, which ranged from 6 to 29 months in storage. Patients' disease activity was assessed using the disease activity score 28 joints (DAS28) criteria at each visit to the clinic with C-reactive protein levels (CRP) weighted as normal when <0.5 mg/dl. The study was conducted in accordance with the declaration of Helsinki and was approved by the local ethics committee. Informed consent was obtained from all patients before study commencement.

Determination of IFX levels and ADAb

IFX serum levels and ADAb were measured following IFX therapy using ELISA assays [Promonitor® IFX Kit (Progenika Biopharma, Spain)] following strict adherence to the manufacturer's version 2 guidelines for use. Serum sample absorbances (OD) were analyzed using the Analysis Software Solutions (MyAssays, Ltd. 2009). IFX levels were considered detectable when >0.035 µg/ml, in compliance with the manufacturer's cut-off values. Similarly, IFX-ADAb was considered present when >2UA/ML.This assay fully conformed to FDA (Federal Drug Administration) and EMA guidelines (European Medicament Agency).

ACPA and RF

An enzyme-linked immunosorbent assay (ELISA) test was used to measure serum autoantibodies at baseline and post-IFX. All samples from each patient were tested simultaneously. The autoantibodies measured in this study included ACPA IgG, IgM and IgA and IgM RF. ACPA IgG was determined using a commercial (ELISA) (Axis Sheild Diagnostics, UK), while CCP2-coated ELISA plates (Eurodiagnostica, Malmo, Sweden) were employed together with isotype-specific peroxidase-conjugated sheep anti-human antibodies (Binding Site, UK) to measure ACPA IgA and IgM. Cut-off values were calculated as the mean ± 3SD from five healthy negative controls. Arbitrary units (AU) of binding were calculated using a standard curve constructed from high positive control sera..

RF IgM was determined with an "in-house" test. This test is based on the binding of RFs to rabbit IgG. A 96-well ELISA clear plates (R&D Systems, USA) were half-coated overnight at room temperature with rabbit IgG (1µg/mL) in Bicarbonate Buffer (0.06 M, pH 9.6), the remaining half of plates were only coated with Bicarbonate Buffer and used for background. Sera from patients and controls at concentration 1:200 diluted in PBS-T-BSA, was added to the plate. After washing, HRP-conjugated sheep anti-human IgM (The Binding Site Group, UK) diluted at 1:3200 in PBS-T-BSA was used for RF IgM detection. RF IgM was detected using the same method described above for IgM and IgA anti-CCP autoantibodies.

Statistical analysis

Descriptive statistics included mean, standard deviation (SD), median and interquartile range (IQR). Frequency data were compared by the Pearson's chi-square. Differences in quantitative values between groups were analysed using student's t- or the Mann-Whitney test. Variation in RF and ACPA levels was analysed by means of the Wilcoxon rank-sum test for paired data. Statistically significant differences were considered when the p-value was less than 0.05. All analyses were performed using SPSS v. 17.0 (SPSS, Chicago, Illinois, USA).

Results

Baseline demographic and clinic characteristics of patients

As shown in Table 1, the majority of patients included in the study were female, 84%, with a mean age 50 (SD+/-12) years. Twelve (63%) patients were taking prednisone and 15 (89%) were taking a synthetic disease-modifying anti-rheumatic drug (DMARD) at the time of starting treatment with IFX. Disease was active in all patients under the DAS28 at the time of starting treatment with IFX. At baseline, fourteen (74%) patients were found positive for RF IgM. Sixteen (84%), 18 (95%) and 10 (53%) patients tested positive for anti-CCP IgM, IgG and IgA, respectively (Table II). Serum samples taken following IFX therapy revealed eight (42%)

Table I. Baseline clinical variables.

Baseline	All patients	IFX detectable (n=8)	IFX undetectable (n=11)	p-value
Sex (women) n (%)	16 (84)	7 (88)	9 (82)	0.73
Age at diagnosis, mean (SD)(range)	50 (12)(22-65)	53 (9.1)(39-65)	47 (14)(22-65)	0.43
Age at IFX start, mean (SD)(range)	58 (13)(23-79)	62 (12)(44-79)	55 (14)(23-76)	0.28
Patients with concomitant DMARD n (%)	15 (79)	7 (88)	8 (72)	0.43
Concomitant MTX n (%)	10 (53)	3 (37)	7 (63)	0.25
Concomitant LEF n (%)	3 (16)	2 (25)	1 (10)	0.34
Concomitant AZA n (%)	2 (10)	2 (25)	0	0.07
Concomitant CS n (%)	12 (63)	6 (75)	6 (54)	0.36
Mean DAS28 score (SD) (range)	5.5 (0.94)(3.4-7.1)	5.7 (0.99)(4.4-7.1)	5.3 (0.91)(3.4-6.1)	0.59

IFX: infliximab; SD: standard deviation; DMARD: disease-modifying anti-rheumatic drugs; MTX: methotrexate; LEF: leflunomide; AZA: azathioprine; CS: corticosteroid; DAS28: Disease Activity Score (28 joints).

Table II. Baseline rheumatoid factor and anti-citrullinated protein antibodies isotypes.

Baseline	All patients	IFX detectable (n=8)	IFX undetectable (n=11)	p-value
Positive RF IgM n (%)	14 (74)	7 (88)	7 (63)	0.6
Mean (SD) (range)	115 (138)(12-401)	90 (149)(12-400)	140 (140)(12-400)	0.45
Positive ACPA IgM n (%)	16 (84)	7 (88)	9 (82)	0.73
Mean (SD) (range)	65 (21)(28-95)	64 (24)(28-95)	66 (19)(45-90)	0.91
Positive ACPA IgG n (%)	18 (95)	8 (100)	10 (91)	0.38
Mean (SD) (range)	127 (121)(5.4-349)	130 (110)(13-280)	130 (130)(5.4-350)	0.96
Positive ACPA IgA n (%)	10 (53)	6 (75)	4 (36)	0.09
Mean (SD) (range)	80 (22)(58-110)	80 (24)(58-110)	79 (23)(58-110)	0.76

IFX: infliximab; RF: rheumatoid factor, ACPA: anti-citrullinated protein antibodies; SD: standard deviation.

of the 19 patients presenting detectable IFX levels with the remaining 11 (58%) testing negative. There were no statistically significant differences in baseline clinical and autoantibody serum levels between groups (Tables I-II).

### IFX serum levels, ADAb and disease activity following IFX therapy

As shown in Table III there were no significant differences in IFX treatment duration or average dosage between the IFX-detectable and undetectable groups. IFX-detectable patients received a mean dosage (SD) of 3.5 mg/kg of body weight against 3.2 mg/ kg in the IFX-undetectable group (p=0.33). All patients with detectable IFX levels were undergoing concomitant synthetic DMARD therapy. Of the remaining 11 patients with undetectable IFX levels, five (45%) were on synthetic DMARD (p=0.01).

Five of the 19 patients were either in remission or low disease activity sta-

tus under DAS28 (DAS28<3.2). Four (80%) of these patients had detectable IFX levels with one (20%) testing IFX undetectable (p=0.04), the latter was undergoing treatment with 7.5 mg prednisone.

All patients were suffering disease with 99 months being the mean time from diagnosis to the start of treatment (SD=75; range: 12-276), regardless of group [IFX detectable (110 SD=69; range: 14–200) and IFX undetectable (93 SD=87; range: 13–270; p=0.59)]. There was no correlation between mean treatment time in months with IFX levels, Spearman correlation r=0.19 (95%CI: -0.31–0.66; p=0.45), nor with ADAb presence, Spearman correlation r=0.13 (95% CI: -0.36–0.56; p=0.57). Nine (47%) of our patients presented ADAb. Eight (72%) of the 11 patients with undetectable IFX had ADAb compared to only one of the 8 IFX-detectable patients (p=0.009), although it is
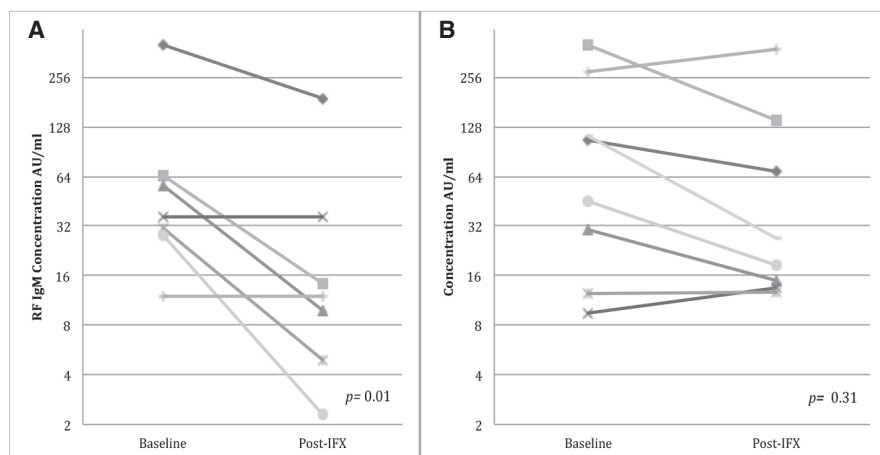
Fig. 1. RF IgM in IFX-detectable (**A**) *vs*. IFX-undetectable patients (**B**). At baseline and post-IFX treatment.
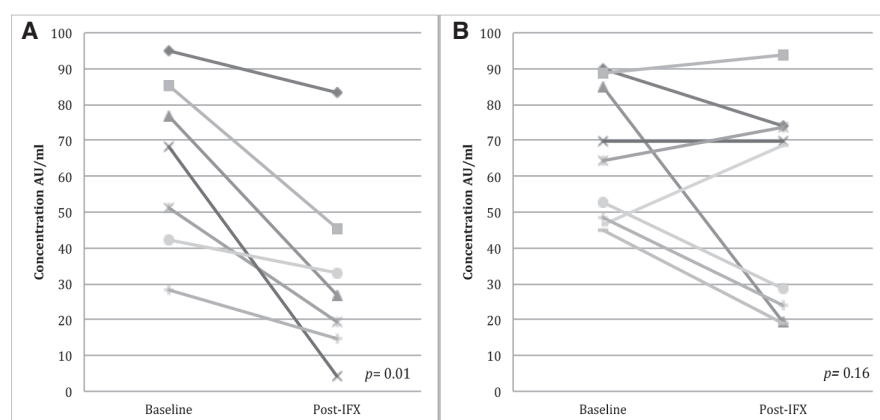
Fig. 2. ACPA IgM in IFX-detectable (**A**) *vs*. IFX-undetectable patients (**B**). At baseline and post-IFX treatment.

Table III. Clinical variables following IFX-therapy.

Post-IFX	IFX detectable (n=8)	IFX undetectable (n=11)	*p*-value
Mean time of IFX treatment in months (SD) (range)	20 (6.8)(8-26)	19 (7.2)(6-29)	0.71
Mean dose of IFX (SD) (range)	310 (64)(200-400)	260 (50)(200-300)	0.11
Patients on concomitant DMARDs (%)	8 (100)	5 (45)	0.01
Concomitant MTX (%)	3 (37)	3 (27)	0.63
Concomitant LEF (%)	4 (50)	2 (18)	0.14
Concomitant AZA (%)	1 (12)	0	0.22
Concomitant CS (%)	4 (50)	5 (45)	0.84
Mean DAS28 (SD) (range)	4.1 (1,6)(2,3-6,6)	5 (1.4)(1.2-6-4)	0.23
Patients in remission or low disease activity DAS28 (%)	4 (50)	1 (9)	0.04
Mean IFX levels (SD) (range)	3 (4.5)(0.28-13)	0.03 (0.008)(0.01-0.04)	<0.05
Mean IFX ADAb concentration (SD) (range)	2.4 (0.99)(2-4.8)	130 (190)(2-640)	<0.05
Detectable IFX ADAb patients (%)	1	8	<0.05

IFX: infliximab; SD: standard deviation; DMARD: disease-modifying anti-rheumatic drugs; MTX: methotrexate; LEF: leflunomide; AZA: azathioprine; CS: corticosteroid; DAS28: Disease Activity Score (28 joints); ADAb: anti-drug antibodies.

interesting to note that the latter presented low levels of serum IFX. Half of the patients with detectable IFX levels were either in remission or attained low disease activity compared to only one patient with undetectable IFX levels. No differences were observed in mean dose (DE) per kilo between ADAb positive, (3.4± 0.48) and ADAb negative patients (3.2 ±0.29; *p*=0.69).

There were no statistically significant differences in the mean baseline levels of RF isotype IgM and ACPA isotypes IgA, IgM and IgG between the patients with detectable and undetectable IFX levels (*p*=0.6; *p*=0.09; *p*=0.73 and *p*=0.38, respectively).

ACPA isotypes and RF variation before and after IFX treatment
Autoantibody levels were determined before and after IFX therapy (Table II). All IFX-detectable patients 8/8 and 10 out of 11 IFX-undetectable patients tested positive for ACPA IgG in the baseline sample (p=0.38). Patients with IFX detectable levels presented a statistically significant reduction in ACPA IgG concentration (*p*=0.007) following treatment. Conversely, changes in ACPA IgG were not significant in patients with IFX undetectable levels (*p*=1.0) (Fig. 1A-B).
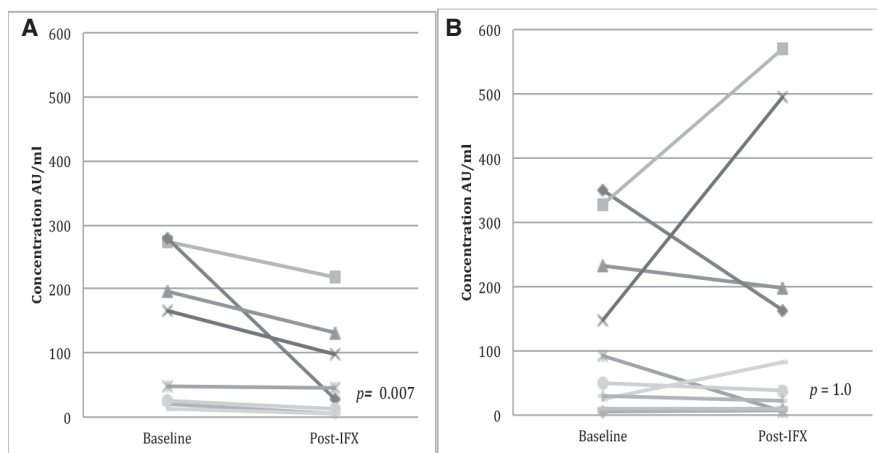At baseline, 7 (88%) of the 8 IFX-detectable patients and 9 (82%) of the 11 IFX- undetectable patients tested positive for ACPA IgM (*p*=0.73). Those IFX-detectable patients underwent a significant decrease in ACPA IgM levels following IFX therapy (*p*=0.01). Such changes in patients with IFX-undetectable levels were found to be not significant (*p*=0.16) (Fig. 2A-B). At baseline, 6 (75%) of the 8 IFX-detectable and 4 (25%) of the IFX-undetectable patients tested positive for ACPA IgA (*p*=0.09). Of these 10 ACPA IgA positive patients, two IFX-detectable and one IFX-undetectable patients went on to test serum negative following treatment.
Those IFX-detectable patients saw a significant reduction of ACPA IgA following IFX treatment (*p*=0.03). Such changes were found to be not significant in the IFX-undetectable group (*p*=0.44) (Fig. 3A-B).
Regarding RF IgM, a significant reduction was observed in the IFX-detectable group following IFX treatment (*p*=0.01) whereas no significant changes to concentrations of this antibody were observed in the IFX-undetectable group (*p*=0.31) (Fig. 4A-B).

Discussion
The main objective of this study was to investigate the relationship between se-

Fig. 3. ACPA IgG in IFX-detectable (**A**) *vs.* IFX-undetectable patients (**B**). At baseline and post-IFX treatment.
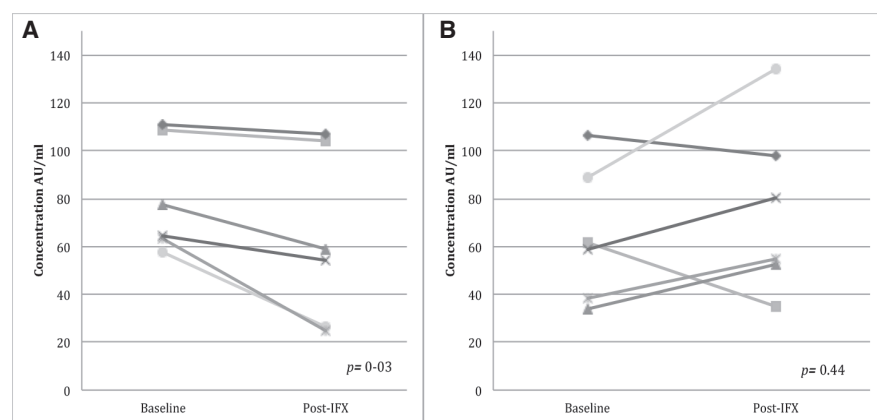
Fig. 4. ACPA IgA in IFX-detectable (**A**) *vs.* IFX-undetectable patients (**B**). At baseline and post-IFX treatment.

rum infliximab (IFX) levels and changes of RF and ACPA levels in patients with RA. We found that there was a significant decrease in these autoantibodies in patients with detectable levels of IFX following therapy, supporting the hypothesis that anti-TNF modifies RF and anti-CCP antibodies as previously reported (9, 10, 13, 14).

Secondary findings included a statistically significant correlation between detectable serum IFX and clinical response to treatment. Half of the patients showing detectable IFX levels were in remission or low disease activity following therapy as opposed to only one patient with IFX undetectable levels as we have previously reported (15). Our data also reveals MTX concomitant use and adequate IFX levels seem to result in longer states of remission. Other studies have also uncovered the same relationship, perhaps owing to synergistic effects.

We cannot, however, state that the presence of ADAb causes a downregulation of IFX; merely that there is an association between the two. Firstly, the scale of our study is limited and secondly, we considered IFX presence as dichotomic without considering degrees of concentration. Additionally, RA pathogenesis comprises many highly complex interactions among different cells and molecules of the innate and adaptive immune system that might explain this phenomenon. For example, we know that targeting TNF and B cells leads to positive therapeutic outcomes for high numbers of RA patients although the exact pathway as yet remains unclear (13, 16, 17).

It is thought that TNF may regulate B cell antibody production through the TNFR1 and TNFR2 membrane receptors (18). TNF also intervenes in the development of follicular dendritic cells,

essential to the formation of germinal centres in the secondary lymphoid organs, which are essential for antibody production and is where interactions between T cells and B cells normally occur (19, 20). Anolik *et al.* studied the effect of anti-TNF based treatment on the secondary lymphoid organs and on the B cell subpopulation in the peripheral blood of RA patients (21). They studied B cell subpopulations in peripheral blood and carried out immunohistochemistry analyses in tonsil biopsy samples taken from both healthy patients and RA patients who had been treated with etanercept. They found that the proportion of follicular dendritic cells as well as the number and size of germinal centres in the secondary follicles were significantly lower in patients treated with anti-TNF. In peripheral blood, patients treated with anti-TNF showed a significantly lower amount of memory B cells. It may be reasonable to propose that anti-TNF drug effectiveness might be linked to a possible detrimental effect on B-cells (21).

We observed that a higher number of patients with adequate drug concentrations reach the remission stage of the disease, just as it had been observed in other studies (22). As well, this study found that there is an inverse relationship between ADAb presence and free serum concentration of the drug. The most revealing result was that the drop in concentration of ACPA and RF in RA patients who have been treated with IFX is associated with the presence of adequate serum concentrations of the drug. Also the assessment and monitoring of serum levels of anti-TNF therapies may be useful to explain the lack or loss of response to treatment in some patients (23). This finding comes to support the role played by anti-TNF-α in the modification of the disease, not only as anti-cytokine therapy, but by indirectly showing its effects on B cells and perhaps in the development of autoantibodies. In conclusion, our data support the hypothesis that anti-TNF antagonists downregulate autoantibody levels in RA patients. Larger-scale studies need to be performed to establish RF and ACPA presence as therapeutic predictive factors.

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